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Bioaccumulation and ecotoxicological responses of juvenile white seabream (*Diplodus sargus*) exposed to triclosan, warming and acidification☆

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ABSTRACT

Triclosan (TCS) is a synthetic microbial compound widely used in the formulation of various personal care products. Its frequent detection in marine ecosystems, along with its physical and chemical properties, suggest that TCS can be highly persistent, being easily bioaccumulated by biota and, therefore, eliciting various toxicological responses. Yet, TCS's mechanisms of bioaccumulation and toxicity still deserve further research, particularly focusing on the interactive effects with climate change-related stressors (e.g. warming and acidification), as both TCS chemical behaviour and marine species metabolism/physiology can be strongly influenced by the surrounding abiotic conditions. Hence, the aim of this study was to assess TCS bioaccumulation and ecotoxicological effects (i.e. animal fitness indexes, antioxidant activity, protein chaperoning and degradation, neurotoxicity and endocrine disruption) in three tissues (i.e. brain, liver and muscle) of juvenile *Diplodus sargus* exposed to the interactive effects of TCS dietary exposure (15.9 $\mu g\,kg^{-1}$ dw), seawater warming ($\Delta T°C = +5$ °C) and acidification ($\Delta pCO_2 \sim +1000$ µatm, equivalent to $\Delta pH = -0.4$ units). Muscle was the primary organ of TCS bioaccumulation, and climate change stressors, particularly warming, significantly reduced TCS bioaccumulation in all fish tissues. Furthermore, the negative ecotoxicological responses elicited by TCS were significantly altered by the co-exposure to acidification and/or warming, through either the enhancement (e.g. vitellogenin content) or counteraction/inhibition (e.g. heat shock proteins HSP70/HSC70 content) of molecular biomarker responses, with the combination of TCS plus acidification resulting in more severe alterations. Thus, the distinct patterns of TCS tissue bioaccumulation and ecotoxicological responses induced by the different scenarios emphasized the need to further understand the interactive effects between pollutants and abiotic conditions, as such knowledge enables a better estimation and mitigation of the toxicological impacts of climate change in marine ecosystems.
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1. Introduction

The production and use of pharmaceutical and personal care products (PPCPs) has substantially increased over the last few decades (Arpin-Pont et al., 2016; Rodríguez-Mozaz et al., 2017). As a result, these chemical contaminants are persistently discharged in

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marine ecosystems (particularly estuaries and coastal areas located near highly urbanized and industrialized areas), through municipal wastewater treatment plants (WWTPs), as well as run offs from agricultural and aquaculture areas (Gros et al., 2012; Gaw et al., 2014). Hence, PPCPs have been frequently detected in marine coastal waters (concentrations ranging from 0.01 ng L^{-1} up to 0.23 mg L^{-1}; Gaw et al., 2014; Arpin-Pont et al., 2016; Rodríguez-Mozaz et al., 2017) and biota (e.g. concentrations ranging from ~1 ng g^{-1} dry weight, dw, for some pharmaceutical active compounds, such as carbamazepine, up to 3600 ng g^{-1} lipid weight, lw, for some preservatives like parabens; Vandermeersch et al., 2015). Yet, PPCPs were only recently regarded as environmental pollutants with potential risks to non-target aquatic species, as well as to humans, and despite some of these compounds have been already included in the list of priority substances in the field of water policy (EC, 2008), their levels in the environment (and seafood) still require regulation.

Triclosan (TCS, 5-chloro-2-(2,4-dichlorophenoxy)phenol) is a synthetic compound widely used in the formulation of various personal care products (e.g. toothpastes, soaps, detergents and cosmetics; EC, 2010, 2014) given its broad-spectrum antibacterial and antimycotic action. In 2016, its production/consumption in the above mentioned products was estimated to be 850 tons in Europe, and 4760 tons at a global scale (QYR, 2016). Although the high effectiveness of TCS's removal at WWTPs (~90% of the parent compound; TCS usually does not undergo metabolic alteration, since most TCS-containing products are used externally in the human body, and not ingested), small, but not negligible, amounts of TCS are still discharged into the environment through domestic effluents (EC, 2010; Dhillon et al., 2015). Furthermore, agricultural activities are also an (if not the most) important source of TCS environmental contamination, since this compound is also frequently used in the formulation of agro-fertilizing biosolids (Chalew and Halden, 2009). As a result, TCS has been frequently detected in marine ecosystems (e.g. up to 100 ng L^{-1} and 35 μg kg^{-1} dw in seawater and sediments in contaminated coastal areas with strong river and WWTPs discharges, respectively; EC, 2010; from 2 ng g^{-1} dw up to 507 ng g^{-1} dw in marine species; Álvarez-Muñoz et al., 2015; Vandermeersch et al., 2015). Such environmental levels along with TCS chemical properties (i.e. its lipophilic nature) suggest that this pollutant can be highly persistent and easily bioaccumulated by marine species (Dhillon et al., 2015). Apart from its negative effect on microbial resistance, which has been intensively described over the last years (e.g. Bailey et al., 2009; EC, 2010; Jutkina et al., 2018), several ecotoxicological impacts have also been associated with TCS contamination. Some of the previously reported effects of TCS exposure in aquatic biota include endocrine disruption and neurotoxicity (e.g. Matozzo et al., 2002; Hedrick-Hopper et al., 2015), reduced growth and genotoxicity (Gao et al., 2015), as well as impairments on species' immune system, metabolism and xenobiotic detoxification mechanisms (e.g. Regnault et al., 2016; Mi et al., 2018). Although it is generally assumed that most PPCPs are directly uptaken from water (i.e. through inhalation) and, therefore, most studies using aquatic organisms have focused on water exposure (e.g. Hwang et al., 2014; Rowett et al., 2016; Arnot et al., 2017), TCS chemical attributes (e.g. lipophilicity and logarithm of the octanol-water partition coefficient, log KoW, around 4.8; Dhillon et al., 2015) suggest that diet (i.e. trophic transfer along the food chain) may also represent an important exposure pathway, particularly in predatory fish species (Fair et al., 2009; Zenker et al., 2014; Hedrick-Hopper et al., 2015). Hence, further research is still required to better understand the ecotoxicological impacts of this pollutant to marine organisms, particularly accounting for other compound uptake pathways apart from water exposure.

In parallel with the environmental problematic of pollution, climate change effects (e.g. warming and acidification) in marine ecosystems have also recently raised great concerns among the scientific community and environmental managers/regulators, as changes in seawater abiotic conditions can alter the availability, speciation and toxicity of pollutants (Marques et al., 2010; Maulvault et al., 2016, Sampaio et al., 2018), particularly, ionizable ones, like PPCPs (e.g. Munari et al., 2016; Rowett et al., 2016). Such is the case of TCS, which undergoes protonation and loses its negative charge as the surrounding pH decreases (i.e. ionisation constant, pKa, defined at 8.14; Dhillon et al., 2015), becoming more bioavailable and, thus, toxic (Orvos et al., 2002; Rowett et al., 2016). On the other hand, by affecting marine biotas' physiology and welfare, climate change effects can also potentially compromise the resilience of these species to other stressors, such as chemical contamination (e.g. Rosa et al., 2016; Jesus et al., 2018; Maulvault et al., 2017). Hence, understanding the interactive effects between chemical pollutants and abiotic stressors has become an urgent matter, since climate change effects can already be felt in many geographic locations (e.g. in Ria Formosa coastal lagoon, situated in Olhão, southern Portugal; Barbosa, 2010; Rodrigues et al., 2017) and are expected to worsen in the next 50–100 years, with southern Europe being particularly prone to these impacts (IPCC, 2001, 2014). According to the most recent projections, seawater temperature and pCO_2 levels are expected to rise as high as +5 °C and +1000 CO_2 μatm (i.e. equivalent to −0.4 pH units), respectively, even if efforts are made to maintain the emissions of greenhouse gases at the present levels (scenario RCP 8.5, IPCC, 2014; McNeil and Sasse, 2016), likely surpassing species' acclimation ability (i.e. species' physiological and molecular plasticity; e.g. Madeira et al., 2016a; Araújo et al., 2018). Moreover, increased frequency, duration (chronicity) and intensity of extreme climate events, such as heat waves (that last at least 5 days in a row, subsequently, interfering with the remaining water chemistry parameters), are also expected to occur in a climate change context (IPCC, 2014). These effects are expected to be particularly notorious (or even to be felt in a shorter term) in estuaries and coastal area, which are naturally exhibit pronounced shifts of abiotic conditions in month (according to season) or even day time scales (particularly, in shallow water zones with weak hydrodynamic activity, e.g. inner zones of the lagoon and coastal ponds), due to the influence of season and/or tides, among other factors (Barbosa, 2010; Madeira et al., 2016a,b; Rodrigues et al., 2017). Even though marine fish inhabiting these unstable environments (such as the seabreams, e.g. *Diplodus sargus*) may have developed different ecological strategies to cope with great daily or monthly amplitudes of abiotic conditions, recent evidence suggested that these species are extremely vulnerable to climate change effects as they already live close to their physiological thresholds and have limited acclimation plasticity (e.g. Madeira et al., 2012, 2016a). Hence, climate change effects are expected to have devastating impacts in estuaries and coastal areas, like Ria Formosa, causing biodiversity reduction and great economic losses in the fisheries (e.g. due to decreased juvenile recruitment) and aquaculture sectors (Barbosa, 2010; Rodrigues et al., 2017), since these ecosystems not only are important spawning and nursery areas (and, thus, shelter many bivalve, crustacean and fish species, e.g. *D. sargus*, during their early and most vulnerable life stages), but also house many aquaculture facilities (in which species are also exposed to changes of abiotic conditions, depending on the type of rearing system used).

Within this context, the aim of the present study was to assess the interactive effects of TCS dietary exposure (15.9 μg kg^{-1} dw), seawater warming ($\Delta T^{o}C$ = +5 °C) and acidification (ΔpCO_2 ~ +1000 μatm, equivalent to ΔpH = −0.4 units) in compound bioaccumulation and ecotoxicological responses (animal

condition, antioxidant activity, chaperoning and protein degradation, neurotoxicity and endocrine disruption) in three tissues (muscle, liver and brain) of juvenile white seabream (*D. sargus*), i.e. an economically and ecologically important fish species that typically inhabits coastal areas subjected to strong anthropogenic and climate-related pressures.

2. Materials and methods

2.1. Model species

D. sargus is a fish species commonly found in Southern Europe, namely in Ria Formosa coastal lagoon, i.e. a sensitive marine ecosystem (and natural park; ICNF, 2008) exposed to strong anthropogenic and climate-related pressures (as described in the Introduction section). It is a highly appreciated seafood species (both wild and farmed) among European consumers, thus, being commercially relevant in the fisheries sector (e.g. about 4000 tons of total catches in 2011; FAO, 2018; up to 29% of total catches in Southern Portugal; IUCN, 2018), as well as in aquaculture (though to a lower extent; i.e. 174 tons produced in aquaculture during 2010; FAO, 2018; IUCN, 2018). By being a predator species that feeds on small benthic organisms that are often in direct contact with contaminated sediments, wild *D. sargus* can likely accumulate high levels of chemical contaminants, particularly through dietary exposure (i.e. ingestion of contaminated preys). As for the effects of abiotic stressors, throughout its life cycle, wild *D. sargus* alternates between estuaries and coastal lagoons during spawning and fish early life stages (eggs, larvae and juveniles), and shallow coastal waters (once the adult stage is reached), therefore, being subjected to wide regimes of seawater abiotic conditions (IUCN, 2018). In aquaculture conditions, although *D. sargus* optimal rearing temperature and pH are around ~19 °C and ~8.0 pH units, respectively (e.g. Moretti et al., 2005; Saavedra et al., 2006), farmed specimens are also susceptible to strong variations of seawater parameters, since low volumes of water and shallow water depths are usually used in fish rearing. Yet, the severity of these changes will depend on the type of aquaculture system used in fish rearing, as this species can be reared extensively and semi-intensively (in coastal ponds and lagoons), as well as intensively using land-based installations (with open or semi-closed aquaculture rearing systems) or sea cages (Moretti et al., 2005). Based on this background knowledge, *D. sargus* was selected as model species due its ecological and commercial relevance, i.e., it is a commercially relevant predatory fish species (i.e. placed in the higher levels of the food chain) typically exposed to abiotic stressors and pollution and has a very common life cycle among coastal fish species, therefore, representing a very interesting model species that adequately suited the purposes of understanding the ecotoxicological implications of climate change in marine fish.

2.2. Experimental setup

2.2.1. Control and TCS-contaminated feeds

An experimental non-contaminated feed (CTR feed) with an adequate composition to meet the nutritional requirements of juvenile white seabream was manufactured by SPAROS Lda (Olhão, Portugal) (details on feed composition are presented in *Supplementary Materials 1*_Table S1). Briefly, all powder components were ground (<200 micron; micropulverizer hammer mill, Hosokawa Micron, SH1, The Netherlands), and mixed with fish oil according to the target feed composition (all ingredients used in fish feeds are presented in *Supplementary Materials 1*_Table S1), using a paddle mixer (Mainca RM90, Spain). Then, 25% of deionized water at room temperature was used to further humidify the feed mixture. The feed was subsequently extruded (at 3.0 mm; low-shear extruder, P55, Italplast, Italy) and subsequently dried on vibrating fluid bed dryer (model DR100, TGC Extrusion, France). Then, a 10 kg batch of this non-contaminated feed was also used to perform the enrichment with TCS (i.e. TCS-contaminated feed), by top-coating pellets with a TCS solution ($C_{12}H_7Cl_3O_2$, >98% purity, Sigma-Aldrich; TCS previously solubilized in 5 mL of ethanol and then mixed in fish oil, making up a total volume of 200 mL) using a pressurized spraying container (standard flat-fan nozzle, size 250 micron, pressure 2.1 bar; the low volume of ethanol used to first solubilize TCS was totally volatilized during this step). Considering the lack of previous studies on pharmaceuticals dietary exposure, a TCS nominal concentration of approximately 15 $\mu g\,kg^{-1}$ on a dry weight basis (dw) was selected to assure that TCS bioaccumulation and ecotoxicological responses were induced during the timeline of the trial. As a reference, although such nominal concentration was above the levels frequently found in marine biota inhabiting contaminated coastal areas (i.e. ~7 times higher; Álvarez-Muñoz et al., 2015), this value was still below the lowest observed effect concentration reported in the only available study on TCS dietary exposure in aquatic species, to the best of the authors' knowledge (Hedrick-Hopper et al., 2015). At the beginning and end of the trial, TCS concentration was determined in both experimental feeds in order to assure: i) TCS stability (in TCS-contaminated feed); and ii) no external contamination occurred during feed preparation (in CTR feed).

2.2.2. Specimens collection and acclimation

Juvenile *D. sargus* specimens with similar age (with approximately 200 days of post-hatching period; $n = 168$) and morphometry (total length: 4.0 ± 0.3 cm; total weight 6.1 ± 0.4 g) were reared at the aquaculture pilot station of the Portuguese Institute for the Sea and Atmosphere (EPPO-IPMA, situated in Ria Formosa coastal lagoon, Olhão, Portugal), using the routine hatchery conditions used in Southern European Countries (e.g. Saavedra et al., 2006). Then, juvenile fish were transplanted (September, 2017, which corresponded to the end of the summer season) to the facilities of Laboratório Marítimo da Guia (MARE-FCUL, Cascais, Portugal), where the exposure trial took place. Here, fish were distributed, in a random and equitable way, in 24 rectangular shaped incubating glass tanks (50 L each, total volume) within recirculation aquaculture systems (RAS; i.e. each group of three tanks corresponded to one treatment, and 3 replicate tanks × 8 treatments = 24 tanks in total; see the description of each treatment in section 2.3.) with independent functioning (Fig. 1). To maintain seawater quality, each tank was equipped with: i) protein skimmers (Reef SkimPro, TMC Iberia, Portugal); ii) ultra-violet (UV) disinfection (Vecton 300, TMC Iberia, Portugal); iii) biological filtration (model FSBF 1500, TMC Iberia, Portugal); and iv) chemical filtration (activated carbon, Fernando Ribeiro Lda, Portugal). Furthermore, fish faeces were removed daily in each incubation tank, and a 25% seawater renewal was performed. Ammonia, nitrite and nitrate levels were daily checked through colorimetric tests (Tropic Marin, USA), and kept below detectable levels (except nitrates, which were kept below 2.0 $mg\,L^{-1}$). On a weekly basis, seawater total alkalinity was also measured in every tank according to the methodology of Sarazin et al. (1999), and the carbonate system parameters were calculated through the combination of total alkalinity (AT) and pH (average values obtained for each treatment presented in *Supplementary Materials 1*_Table S2).

Before starting the exposure trial, an acclimation period was carried out during 30 days, with fish being fed with CTR feed (2% average body weight, BW) and kept under the following abiotic conditions: i) temperature = 19.0 ± 0.5 °C; ii) pH = 8.00 ± 0.10 units; iii) salinity = 35 ± 1‰; iv) dissolved oxygen (DO) > 5 $mg\,L^{-1}$;

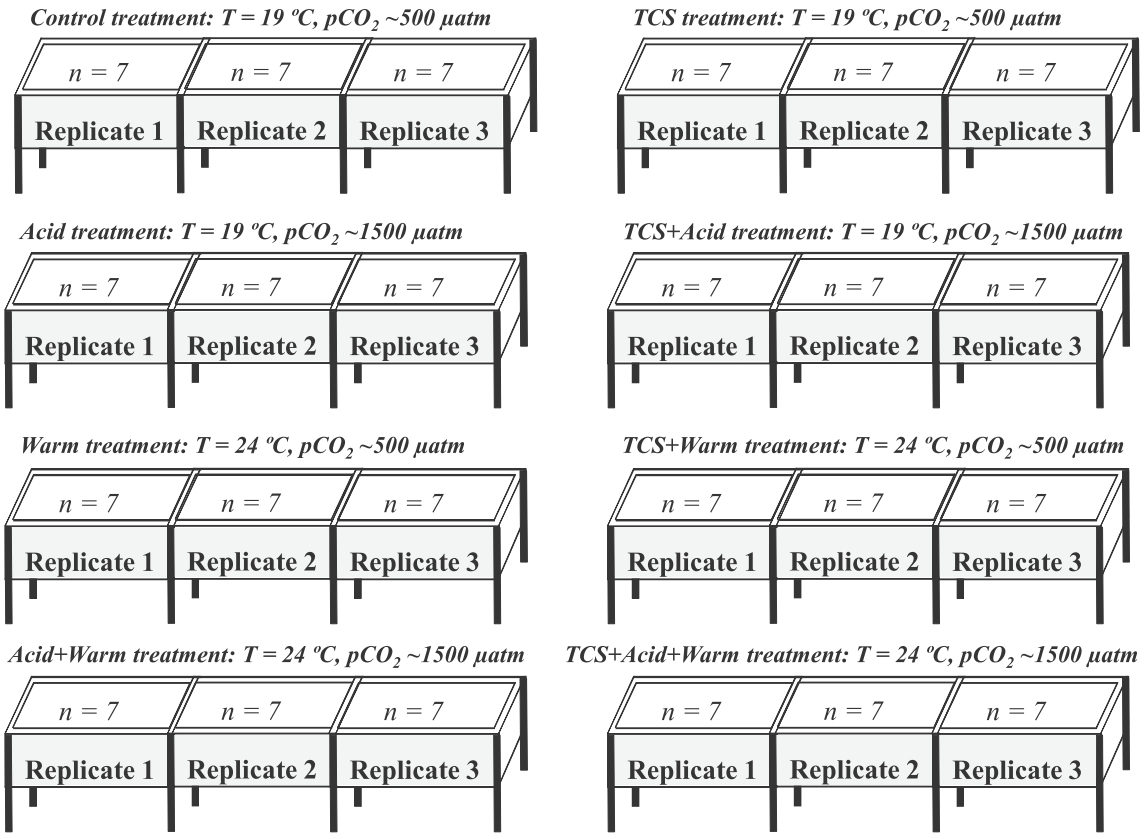
Control treatment: T = 19 ºC, pCO2 ~500 µatm
TCS treatment: T = 19 ºC, pCO2 ~500 µatm
Acid treatment: T = 19 ºC, pCO2 ~1500 µatm
TCS+Acid treatment: T = 19 ºC, pCO2 ~1500 µatm
Warm treatment: T = 24 ºC, pCO2 ~500 µatm
TCS+Warm treatment: T = 24 ºC, pCO2 ~500 µatm
Acid+Warm treatment: T = 24 ºC, pCO2 ~1500 µatm
TCS+Acid+Warm treatment: T = 24 ºC, pCO2 ~1500 µatm
n = 7
Replicate 1
Replicate 2
Replicate 3

Fig. 1. Experimental setup. Abbreviations: TCS – triclosan exposure; Acid – simulated acidification (i.e. pCO_2 ~1500 µatm, equivalent to pH = 7.6 units); Warm – simulated warming (i.e. T = 24 °C).

and v) photoperiod = 14 h light and 10 h dark (the multi-parameter measuring instrument Multi 3420 SET G, WTW, Germany, was used to daily check seawater temperature, pH, salinity and DO). These seawater abiotic conditions were similar to the ones to which fish were exposed to at EPPO, before being transplanted to the experimental tanks, and corresponded to the optimal conditions for juvenile *D. sargus* rearing (Moretti et al., 2005; Saavedra et al., 2006). Temperature and pH were adjusted whenever needed using: i) temperature - an automatic seawater refrigeration system (±0.1 °C; Frimar, Fernando Ribeiro Lda, Portugal), as well as submerged digital thermostats (200 W, V2Therm, TMC Iberia, Portugal); ii) pH - individual pH probes (GHL, Germany) connected to a Profilux 3.1N control system (±0.1 pH units, GHL, Germany), which monitored seawater pH in each tank every 2 s, and adjusted the levels whenever needed, through the injection of either CO_2 (Air Liquide, Portugal; to decrease pH) or CO_2-filtered aeration (Stella 200 air pumps, Aqua One Pro, Aqua Pacific UK Ltd, United Kingdom; to increase pH).

2.2.3. Exposure to TCS, warming and acidification

One week before initiating the trial, seawater temperature and pCO_2 were slowly adjusted (+1 °C and −0.1 pH unit per day), until reaching the simulated climate change conditions (, i.e. 24 °C and ~1500 µatm pCO_2, equivalent to pH = 7.6 units, which corresponded to +5 °C and +1000 µatm pCO_2 in relation to juvenile *D. sargus* optimal rearing conditions, as well as to the seawater conditions that fish were exposed to before transplantation to the experimental tanks; scenario RCP8.5 of IPCC, 2014; McNeil and Sasse, 2016) in the corresponding treatments (Fig. 1). Although the seawater warming and acidification conditions simulated in the present study can be defined as an extreme climate change scenario (i.e. worst-case scenario), the reader should note that these conditions are particularly likely to occur (in a shorter time frame) in shallow water coastal environments with low hydrodynamic activity, that naturally exhibit strong tidal/seasonal abiotic variations and are particularly prone to extreme climate events (e.g. heat waves), and in which some of the projected climate change effects are already visible, as is the case of Ria Formosa (Barbosa, 2010; Rodrigues et al., 2017). Hence, the altered abiotic conditions simulated in the present study can be considered realistic (according to IPCC projections) and ecologically representative. The remaining seawater abiotic conditions (i.e. DO, salinity and photoperiod) were kept as previously described in section 2.2. (i.e. as during the acclimation period). Thus, eight treatments (4 non-contaminated and 4 TCS-contaminated treatments), randomly assigned to each replicate tank, were carried out ($n = 7$ animals per replicate tank of treatment, i.e. a total of 21 animals per treatment; Fig. 1), using a full cross-factorial design: i) Control treatment – 19 °C, 500 µatm pCO_2 and fish daily fed with CTR feed (2% BW); ii) Acid treatment - 19 °C, 1500 µatm pCO_2 (equivalent to pH = 7.6 units) and fish daily fed with CTR feed (2% BW); iii) Warm treatment - 24 °C, 500 µatm pCO_2 and fish daily fed with CTR feed (2% BW); iv) Acid + Warm - 24 °C, 1500 µatm pCO_2 (equivalent to pH = 7.6 units) and fish daily fed with CTR feed (2% BW); v) TCS treatment – 19 °C, 500 µatm pCO_2 and fish daily fed with TCS-contaminated feed (2% BW); vi) TCS + Acid treatment - 19 °C, 1500 µatm pCO_2 (equivalent to pH = 7.6 units) and fish daily fed with TCS-contaminated feed (2% BW); vii) TCS + Warm treatment - 24 °C, 500 µatm pCO_2 and fish daily fed with TCS-contaminated feed (2% BW); and viii) TCS + Acid + Warm - 24 °C, 1500 µatm pCO_2 (equivalent to pH = 7.6 units) and fish daily fed with TCS-contaminated feed (2% BW). Seawater physical and chemical

conditions were daily monitored and adjusted whenever needed, as described above (section 2.2.). No mortality was observed during the 28 day trial. Seawater samples were also collected from each tank at days 0 (beginning) and 28 (end) of the trial, and TCS concentrations were determined to confirm that an external contamination did not occur in non-contaminated treatments, as well as in TCS contaminated treatments (apart from the intended TCS dietary exposure through feed).

2.2.4. Samples collection and preparation

At the end of the trial, 15 fish were randomly collected from each treatment (i.e. 5 fish collected from each of the 3 replicate tanks that composed each treatment), euthanized (i.e. fish immersed for 10 min in a tricaine methanesulfonate, MS222, solution at 2000 mg L^{-1} buffered with sodium bicarbonate, $NaHCO_3$; Sigma-Aldrich, USA for), measured (total length, TL, and weight, W), and dissected (i.e. muscle, brain and liver tissues were collected and weighed).

For TCS determination, due to the amount of sample required by the analytical methodology, two composite samples per tissue and per treatment were performed (i.e. tissues of 5 individuals used in each pool, $n = 2$ pools). Homogenized pooled samples were freeze-dried (−50 °C, 10^{-1} atm of vacuum pressure for 48 h; Power Dry LL3000, Heto, Czech Republic) and kept at −80 °C until further analysis.

To assess fish ecotoxicological responses, tissues of 5 animals from each treatment were individually used. Each tissue (muscle and liver: ~100 mg; brain: ~40 mg) was homogenized with 1.0 mL of phosphate buffered saline (PBS; 3 mM of potassium chloride, KCl, 10 mM of potassium phosphate monobasic, KH_2PO_4 and 140 mM of sodium chloride, NaCl, pH adjusted to 7.40 ± 0.02 units; all reagents from Sigma-Aldrich, Germany), in ice-cold conditions, using a T25 digital Ultra-Turrax device (Ika, Germany). Crude homogenates were then centrifuged in 1.5 mL Eppendorf tubes (15 min, 10.000 *g*, 4 °C), and supernatants were transferred to new Eppendorf tubes, being immediately frozen and kept at −80 °C until further analyses.

2.3. TCS determination in seawater, feeds and fish tissues

For TCS determination in liquid samples (i.e. seawater), the protocol followed was based on the methodology described by Castro et al. (2018), with some adjustments. Briefly, 4.0 mL of sample was placed in 10 mL conic glass tube, and 100 μL of Chrysene D-12 at 0.5 mg L^{-1} were added (solution pH adjusted to ≥ 10 pH units, using droplets of potassium carbonate, K_2CO_3, 23%, analytical grade, Panreac Química SA). Then, a mixture of 85 μL of carbon tetrachloride (CCl_4; 99.9% purity, Panreac Química), 800 μL of acetonitrile and 125 μL of acetic anhydride was rapidly transferred to the 10 mL tube. Each tube was vortexed for 1 min and centrifuged at 1500 *g* for 3 min. Then, 50 μL of end-phase was transferred to an injection vial containing 10 μL of methyltriclosan D-3 at 2 mg L^{-1}, and 1 μL of this mixture was injected into the gas chromatography–mass spectrometry (GC-MS) system.

In solid samples (i.e. feeds and fish tissues), a combined QuEChERS (quick easy cheap effective rugged safe) and DLLME (dispersive liquid-liquid micro extraction) procedure was first carried out following the methodology of Castro et al. (2018) with some modifications. Briefly, 500 mg of feed or freeze-dried tissue sample was weighted to 40 mL dark glass tubes, and 50 μL of internal standard (IS1) was added (Chrysene D_{-12}, 3 mg L^{-1}; 98 atom %, Sigma-Aldrich). After 15 min, samples were hydrated with deionized water (5 mL), and 4 mL of acetonitrile (MeCN, ≥ 99.9% purity, Honeywell Riedel-de Haën ACS), 0.5 g of NaCl (≥99.9% purity Sigma-Aldrich) and 2 g of anhydrous magnesium sulphate ($MgSO_4$, ≥99.5%, Sigma-Aldrich) were added. Sample tubes were vortexed for 30 s, placed in a round shaker for 30 min and, then, centrifuged again (5 min at 4000 *g*). The extracts obtained were used as a dispersive solvent for DLLME. Hence, to 1 mL of MeCN extract obtained, 50 μL of internal standard (IS2; methyl-triclosan D3, 2 mg L^{-1}, Sigma-Aldrich), 60 μL of CCl_4, and 125 μL of acetic anhydride ($C_4H_6O_3$, ≥99% purity, Sigma-Aldrich) were added. This mixture was quickly transferred to a 10 mL screw cap glass tubes with a conical bottom containing 3 mL of deionized (pH ≥ 10 units, adjusted with K_2CO_3 23% droplets). Tubes were manually shaken for a few seconds and centrifuged (3 min at 4000 *g*). The settled volume (60 ± 5 μL) was transferred to an injection vial with 10 μL of methyltriclosan D-3 at 2 mg L^{-1}, and 1 μL of sample was injected in the GC–MS system.

GC-MS analyses were carried using an Agilent gas chromatograph 6890 (Little Falls, DE, USA) equipped with an electronically controlled split/splitless injection port and interfaced to a MSD-5975B mass-selective detector. GC separation was conducted on a DB-5MS column (30 m × 0.25 mm i.d. 0.25 μm film thickness; J&W Scientific, Folsom, CA, USA), using helium as the carrier gas (1 mL min^{-1} of constant gas flow). Injections were made in splitless mode (purge-off time: 60 s) at 250 °C, and the following oven temperature programme (with 28 min of total run time) was carried out: 1 min hold at 90 °C, temperature ramped at 20 °C $minute^{-1}$ until 150 °C, 5 °C $minute^{-1}$ between 150 °C and 225 °C, and then 20 °C $minute^{-1}$ between 225 °C and 300 °C, 5.25 min hold. The following mass spectrometric parameters were set: 70 eV energy electron ionisation, 230 °C of ion source temperature, and 150 °C of mass spectrometry (MS) quadrupole temperature. Selective ion monitoring (SIM) mode was employed and each analyte was quantified using the peak area of one target ion and two qualifier ions. Data collection/processing and GC-MS control was performed on the software Agilent Chemstation. SIM parameters, retention times of analytes, and validation parameters of the methodology are shown in *Supplementary Material_1*_Table S3.

2.4. D. sargus ecotoxicological responses

2.4.1. Fitness indexes (K, HSI and BB_{ratio})

Animal condition was determined by calculating the Fulton's K index (Ricker, 1975), using fish weight (W) and total length (TL):

$$K = 100 \times \frac{W\,(g)}{TL^3(cm)}$$

Organ condition (liver and brain) was also assessed by calculating the hepatosomatic index (i.e. HSI; Diniz et al., 2009) and brain to body mass ratio (i.e. BB_{ratio}; Maulvault et al., 2017) using the following equations:

$$HSI = \frac{Liver\ weight\ (g)}{W\,(g)} \times 100$$

and,

$$BB_{ratio} = \frac{brain\ weight\ (g)}{W\,(g)} \times 100$$

2.4.2. Molecular biomarkers

Eight molecular biomarkers (of exposure and/or effect) were selected to assess distinct biological effects (endpoints) induced by TCS dietary exposure, warming and acidification, at the tissue level (Table 1). These molecular biomarkers have been widely employed in ecotoxicological studies, being considered as reliable and suitable to assess the effects of xenobiotics exposure, including TCS

Table 1
Summary of selected molecular biomarkers and the corresponding methodologies used.

Biomarkers	Category	Tested ecotoxicological response	Type of methodology used	Methodology references
Catalase (CAT) activity	Biomarker of effect	Oxidative stress	Enzymatic assay	Johansson and Borg (1988); Maulvault et al. (2017)
Superoxide dismutase (SOD) activity	Biomarker of effect	Oxidative stress	Enzymatic assay	Sun et al. (1988); Maulvault et al. (2017)
Glutathione S-transferase (GST) activity	Biomarker of effect	Oxidative stress and xenobiotic detoxification phase II	Enzymatic assay	Habig et al. (1974); Maulvault et al. (2017)
Lipid peroxidation (LPO)	Biomarker of effect	Oxidative stress and cellular damage	TBARS method	Uchiyama and Mihara (1978); Madeira et al. (2016a)
HSP70/HSC70 proteins content	Biomarker of effect	Chaperoning, heat shock response	Indirect ELISA	Njemini et al. (2005); Maulvault et al. (2017)
Total Ubiquitin (Ub) content	Biomarker of effect	Protein degradation and DNA repair	Direct ELISA	Madeira et al. (2014)
Acetylcholinesterase (AChE) activity	Biomarker of effect and exposure	Neurotoxicity	Enzymatic assay	Ellman et al. (1961); Maulvault et al. (2017)
Vitellogenin (VTG) content	Biomarker of exposure	Endocrine disruption and reproduction	Direct ELISA	Denslow et al. (1999); Diniz et al. (2009)

(e.g. Matozzo et al., 2002), as well as of climate change-related effects (e.g. Rosa et al., 2016; Jesus et al., 2018; Maulvault et al., 2017, 2018a). Table 1 presents a summary of the selected biomarkers (and the corresponding ecotoxicological response tested), as well as the different methodologies used (further details regarding these methodologies have been previously presented in Maulvault et al., 2017 and Maulvault et al., 2018a). All biomarker values were normalized using the total protein content of each sample (i.e. results presented in mg of protein), determined through the Bradford assay (Bradford, 1976).

All protocols used for both enzymatic and protein quantification assays were adapted to 96-well microplates (microplates from Nunc-Roskilde, Denmark, used in all assays), and carried out in triplicate, and read in a Multiskan Go 1510 microplate reader (ThermoFisher Scientific, USA). All reagents used were of pro analysis grade or higher.

2.5. Statistical analyses

For the different studies variables, differences between treatments were investigated using Generalized Linear Mixed Models (GLMM). Selection for best model was made using Akaike Information Criterion (AIC), i.e.: a Gamma distribution was used to analyse TCS tissue concentration data, whereas a Gaussian distribution was used for fish morphometric (W, TL) and ecotoxicological data (K, HSI and BB_{ratio}, as well as tissue biomarker responses), with replicate tank as random effect. Model assumptions, namely independence and absence of residual patterns, were verified by plotting residuals against fitted values and each covariate in the model. The summary of GLMM results is presented in *Supplementary Materials*, Tables S4-S6. The post-hoc Tukey test was also carried out for multiple comparisons (results are presented in Figs. 2–6). Finally, Pearson correlation coefficients between variables were also calculated. Statistical analyses were performed at a significance level of 0.05, and using the R Core Team 2017 software. Data exploration and model validation used the R library from Highland Statistics (Zuur et al., 2009).

3. Results

3.1. TCS concentration in feeds and seawater samples

An average TCS concentration of $15.9 \pm 2.9\ \mu g\ kg^{-1}$ dw was obtained in TCS-contaminated feed throughout the trial, whereas TCS was not detected in CTR feed (i.e. CTR feed concentration $<0.009\ \mu g\ kg^{-1}$). In the beginning of the trial (day 0), seawater samples from all treatments revealed TCS below LOD (i.e. concentration $<0.050\ \mu g\ L^{-1}$). Yet, at day 28, quantifiable and similar TCS concentrations were found in contaminated treatments (average concentration $= 0.44 \pm 0.10\ \mu g\ L^{-1}$; Fig. 2A), while TCS levels remained below LOD in the Control treatment.

3.2. Fish morphometry and TCS tissue bioaccumulation

After 28 days of trial, fish exposed to the reference temperature and pCO_2 levels (i.e. Control and TCS treatments) exhibited significantly lower W and TL than fish exposed to warming alone (i.e. Warm treatment; $p < 0.010$) or in combination with TCS exposure and acidification (i.e. TCS + Acid + Warm treatment; $p < 0.050$; Fig. 3A).

By the end of the exposure trial, none of the tissues of non-contaminated fish (i.e. Control treatment) revealed detectable levels of TCS (i.e. concentration in fish tissues $<0.009\ \mu g\ kg^{-1}$). As for contaminated treatments, TCS concentrations significantly varied among tissues and treatments, with muscle being the primary organ for TCS bioaccumulation regardless of seawater temperature and pCO_2 conditions (e.g. in TCS treatment: $1.781 \pm 0.114\ \mu g\ kg^{-1}$ dw in muscle, $0.168 \pm 0.005\ \mu g\ kg^{-1}$ dw in liver and $0.050 \pm 0.009\ \mu g\ kg^{-1}$ dw in brain; $p < 0.001$; Fig. 2B–D). Furthermore, significantly lower TCS concentrations were found under increased seawater temperature and/or pCO_2 levels, regardless of tissue, with warming overall yielding lower TCS levels in all organs (i.e. TCS + Warm treatment: $0.268 \pm 0.027\ \mu g\ kg^{-1}$ dw in muscle, $0.027 \pm 0.005\ \mu g\ kg^{-1}$ dw in liver and $0.026 \pm 0.007\ \mu g\ kg^{-1}$ dw in brain) compared to the reference seawater temperature conditions or to acidification alone (Fig. 2B–D). Significant negative correlations were obtained between morphometric data and TCS concentration in liver ($r = -0.62$ and $r = -0.55$ and $p < 0.010$ and $p < 0.050$ in W and TL *versus* TCS liver concentration, respectively; Table 2) and brain ($r = -0.60$ and $r = -0.53$ and $p < 0.01$ and $p < 0.050$ in W and TL *versus* TCS brain concentration, respectively; Table 2) of contaminated fish, but not in muscle (i.e. $p > 0.050$; Table 2).

3.3. Ecotoxicological responses

Fish fitness indexes (K, HSI and BB_{ratio}) after 28 days of exposure to the three stressors are presented in Fig. 3B. While K and BB_{ratio} were not significantly affected by TCS, acidification and/or warming (i.e. $p > 0.050$ in Control treatment against all treatments), significantly higher HSI was found in fish exposed to TCS alone or in combination with acidification compared to fish exposed to the three stressors simultaneously ($p < 0.010$ and $p < 0.05$, respectively; Fig. 3B). Moreover, HSI was significantly and positively correlated

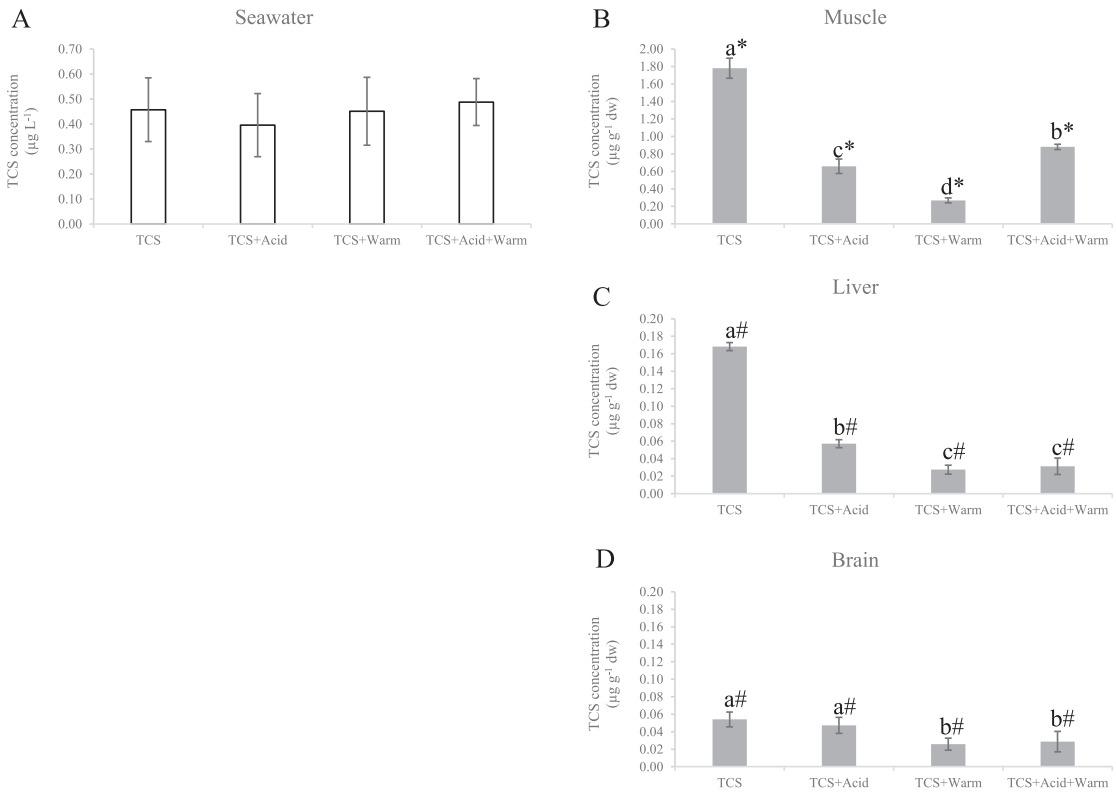

Fig. 2. Triclosan concentration in seawater samples (µg L^{-1}; A) and in fish tissues (µg kg^{-1} dw; B-D) from contaminated treatments after 28 days of TCS dietary exposure. Different letters (a–d) indicate significant differences between treatments (for the same tissue; $p < 0.05$), whereas different symbols (*, # or /) indicate significant differences between tissues (for the same treatment; $p < 0.05$). Abbreviations: TCS – triclosan exposure; Acid – simulated acidification (i.e. pCO_2 ~1500 µatm, equivalent to pH = 7.6 units); Warm – simulated warming (i.e. T = 24 °C).

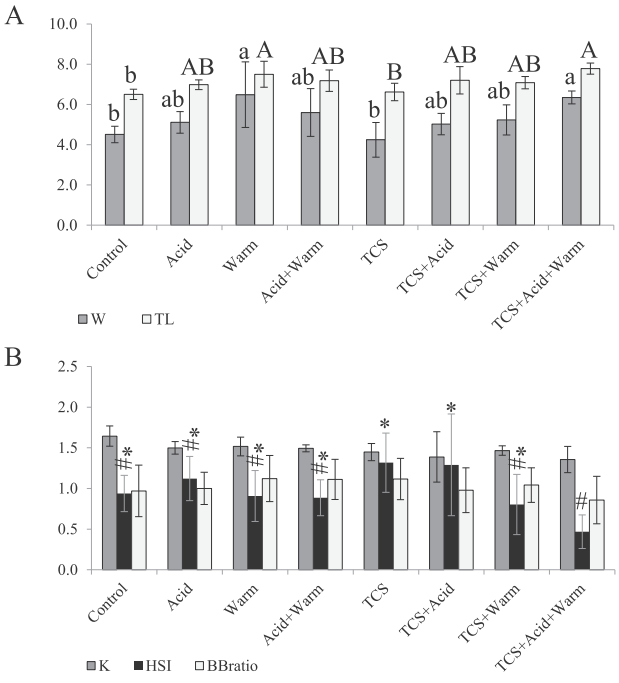

Fig. 3. *D. sargus* total length (TL; cm) and weight (W; g), Fulton's condition index (K), hepatosomatic index (HSI) and brain-to-body mass ratio (BB_{ratio}) after 28 days of trial (mean ± SD; $n = 15$). Different letters (lower case, a-b, in W bars and upper case, A-B, in TL bars) and symbols (* or # in HSI bars) indicate significant differences ($p < 0.05$) between treatments. Abbreviations: TCS – triclosan exposure; Acid – simulated acidification (i.e. pH = 7.6 units); Warm – simulated warming (i.e. T = 24 °C).

with TCS liver concentrations in contaminated fish ($r = 0.46$ and $p < 0.050$; Table 2).

Fig. 4 presents the antioxidant enzymes activity (CAT, SOD and GST) and lipid peroxidation (LPO) in fish tissues. In fish muscle, a significant increase in CAT activity was observed when TCS exposure acted alone (corresponding to a 54.5% increase in relation to Control treatment; $p < 0.050$), but not when combined with the warming and/or acidification (Fig. 4A and *Supplementary Materials 1*_Table S7). Furthermore, in the muscle of contaminated fish, a significant and positive correlation was found between CAT activity and TCS concentration ($r = 0.77$ and $p < 0.001$; Table 2). As for the liver and brain, although CAT activity was not significantly affected by the three stressors acting alone ($p > 0.05$), a significant decrease was observed in the liver of fish co-exposed to TCS and warming (i.e. TCS + Warm treatment), not only in relation to the Control treatment (equivalent to a 38.7% decrease; $p < 0.001$), but also to Warm ($p < 0.001$), TCS + Acid ($p < 0.001$) and TCS + Acid + Warm treatments ($p < 0.001$; Fig. 4B and *Supplementary Materials 1*_Table S7). SOD and GST activities were significantly affected by abiotic stressors, acting alone or combined with each other (i.e. SOD muscle activity decreased around 9% in relation to Control treatment;; $p < 0.050$ and $p < 0.010$, in Control against Warm and Acid + Warm treatments, respectively; GST brain activity increased between 76% and over 100% in relation to Control treatment; $p < 0.050$, $p < 0.001$ and $p < 0.001$ in Control against Acid, Warm and Acid + Warm treatments; Fig. 4D–I and *Supplementary Materials 1*_Table S7). On the other hand, TCS exposure did not affect SOD activity, unless in the liver and brain of fish exposed to the combination of TCS and acidification (corresponding to activity increases of 18.6% and 5.5% in relation to Control treatment, in liver and brain, respectively; $p < 0.001$ and $p < 0.050$, respectively;

Table 2
Pearson correlation coefficients (r) between TCS tissues concentration, animal morphometry, fitness indexes and molecular biomarker responses. In each column, asterisks indicate significant correlations between variables (*: $p < 0.05$; **$p < 0.01$; ***$p < 0.001$). Abbreviations: TW – total weight; TL – total length; K - Fulton's condition index; HSI - hepatosomatic index; BB_{ratio} - brain-to-body mass ratio; CAT – catalase activity; SOD activity inhibition – superoxide dismutase; GST activity – glutathione S-transferase; LPO - lipid peroxidation; HSP70/HSC70 - heat shock proteins concentration; Ub - total ubiquitin concentration; AChE – acetylcholinesterase activity; VTG – vitellogenin concentration; TCS – triclosan exposure; Acid – simulated acidification (i.e. pH = 7.6 units); Warm – simulated warming (i.e. T = 24 °C).

Treatments used in the analysis	Variables	r		
All	W × TL	0.85***		
	K × HSI	0.19		
	K × BB_{ratio}	0.05		
TCS-contaminated treatments		**Muscle**	**Liver**	**Brain**
	[TCS] × W	−0.41	−0.62**	−0.60**
	-	−0.38	−0.55*	−0.53*
	[TCS] × K	0.04	0.08	0.12
	[TCS] × HSI	–	0.46*	–
	[TCS] × BB_{ratio}	–	–	0.37
	[TCS] × CAT	0.77***	−0.02	−0.17
	[TCS] × SOD	−0.20	−0.001	−0.20
	[TCS] × GST	0.26	0.09	0.19
	[TCS] × LPO	0.11	0.17	−0.40
	[TCS] × HSP70/HSC70	−0.17	0.58**	−0.02
	[TCS] × Ub	−0.40	0.47*	0.51*
	[TCS] × AChE	–	–	−0.07
	[TCS] × VTG	–	−0.21	–
All	K × CAT	0.01	−0.16	−0.04
	K × SOD	−0.10	0.09	0.19
	K × GST	0.13	−0.04	−0.07
	K × LPO	−0.02	−0.19	−0.02
	K × HSP70/HSC70	−0.09	0.27	0.40**
	K × Ub	−0.14	−0.33*	0.22
	K × AChE	–	–	0.12
	K × VTG	–	0.37*	–
All	HSI × CAT	–	−0.17	–
	HSI × SOD	–	−0.31	–
	HSI × GST	–	0.24	–
	HSI × LPO	–	0.28	–
	HSI × HSP70/HSC70	–	0.19	–
	HSI × Ub	–	−0.19	–
	HSI × AChE	–	–	–
	HSI × VTG	–	0.11	–
All	BB_{ratio} × CAT	–	–	−0.07
	BB_{ratio} × SOD	–	–	0.05
	BB_{ratio} × GST	–	–	0.28
	BB_{ratio} × LPO	–	–	−0.17
	BB_{ratio} × HSP70/HSC70	–	–	−0.19
	BB_{ratio} × Ub	–	–	−0.04
	BB_{ratio} × AChE	–	–	−0.12
	BB_{ratio} × VTG	–	–	–

Fig. 4E–F and *Supplementary Materials 1*_Table S7). Similarly, TCS exposure did not affect GST activity, except in the muscle and liver of fish co-exposed to TCS and warming (i.e. corresponding to a decrease of 46.8% and 69.6% in relation to Control treatment in muscle and liver, respectively; $p < 0.001$ in both cases; Fig. 4G–H, and *Supplementary Materials 1*_Table S7). It should be further noted that significantly higher GST activity was also found in TCS + Acid treatment compared to TCS + Warm treatment (in muscle and liver; $p < 0.001$ in both cases), as well as to TCS and TCS + Acid + Warm treatments (in liver; $p < 0.001$ and $p = 0.019$, respectively; Fig. 4G–H). Overall, TCS acted alone or in combination with acidification induced significantly higher LPO in fish muscle and liver (i.e. average increase of over 100% in relation to Control treatment, in both tissues), but not in the brain ($p > 0.050$; Fig. 4J–L and *Supplementary Materials 1*_Table S7).

HSP70/HSC70 and Ub contents in the different fish tissues are shown in Fig. 5. Contrasting the trends mentioned above, in the absence of TCS (i.e. in non-contaminated fish), abiotic stressors only affected HSP70/HSC70 content in fish liver, resulting in a significant increase in Acid treatment (i.e. equivalent to 63.2% increase in relation to Control treatment; $p < 0.001$), whereas Acid + Warm treatment significantly inhibited the synthesis of these proteins (i.e. equivalent to 74.0% decrease in relation to Control treatment; $p < 0.001$; (Fig. 5A–C and *Supplementary Materials 1*_Table S7). Conversely, TCS alone or combined with acidification significantly lowered HSP70/HSC70 contents in fish muscle (corresponding to a 44.6% and 56.6% decrease in TCS and TCS + Acid treatments, respectively, relation to Control treatment; $p < 0.001$ in both cases), but such effect was reversed when the three stressors acted simultaneously (i.e. in TCS + Acid + Warm treatment; corresponding to an increase of 66.5% in relation to Control treatment; $p < 0.001$; Fig. 5A and *Supplementary Materials 1*_Table S7). In fish liver, significant effects were only found when TCS was combined with abiotic stressors (i.e. decreases in HSP70/HSC70 contents between 36.6% in TCS + Warm treatments and 76.6% in TCS + Acid treatment, relation to the Control treatment; $p < 0.010$ and $p < 0.001$, respectively; Fig. 5B and *Supplementary Materials 1*_Table S7). No significant differences were observed in HSP70/HSC70 brain contents ($p > 0.050$; Fig. 5C and *Supplementary Materials 1*_Table S7). A significant and positive correlation was found between HSP70/HSC70 and TCS contents in fish liver ($r = 0.58$ and $p < 0.001$), as well as between HSP70/HSC70 and K in the brain ($r = 0.40$ and $p < 0.001$; Table 2). As for Ub content, warming combined with acidification or with TCS significantly increased Ub

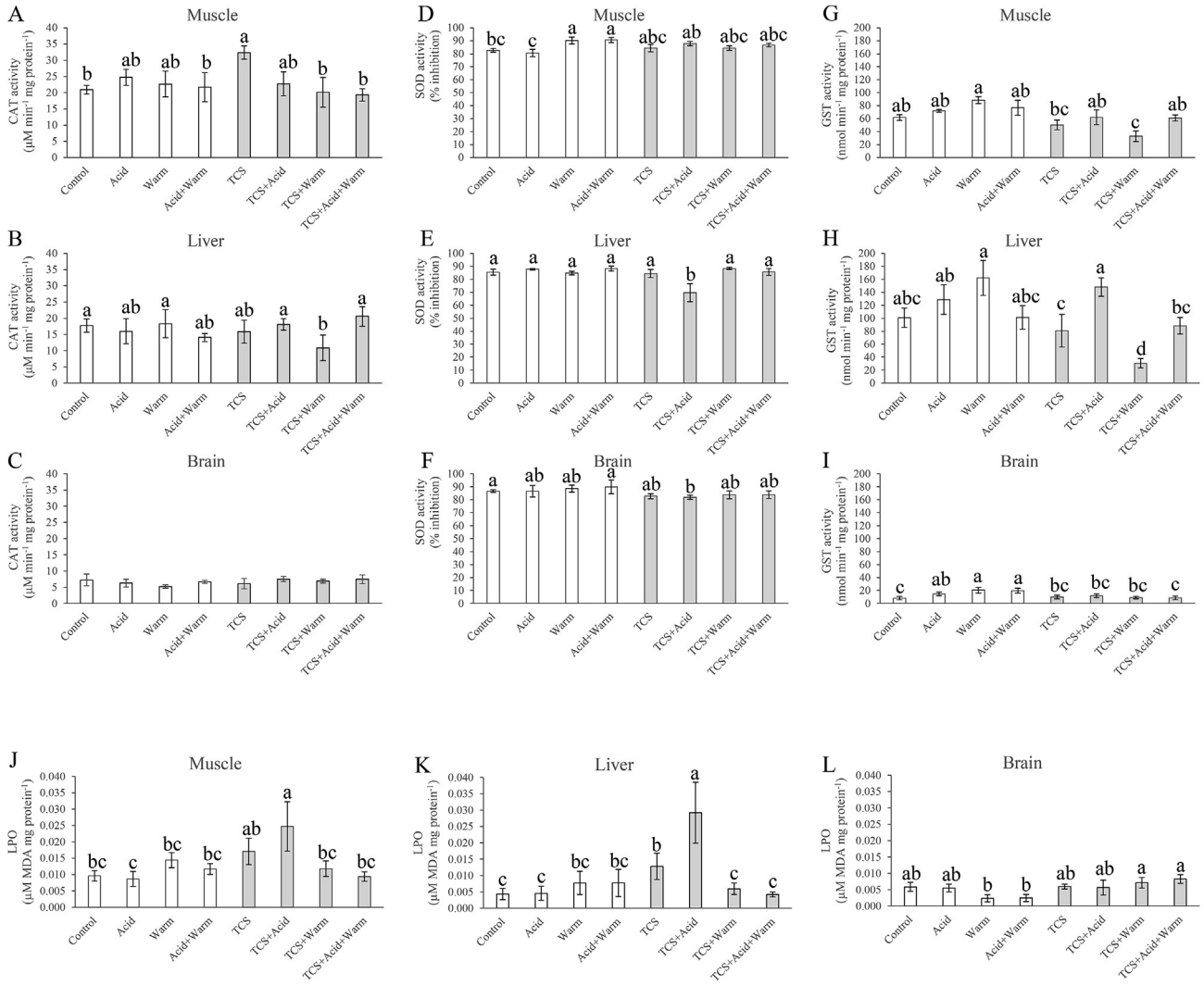

Fig. 4. Antioxidant activity (CAT, μM min $^{-1}$ mg^{-1} protein; SOD, % inhibition; GST, nmol min $^{-1}$ mg^{-1} protein) and lipid peroxidation (expressed as MDA concentration; μM mg^{-1} protein) in muscle (A, D, G, J), liver (B, E, H, K) and brain (C, F, I, L) of *D. sargus* after 28 days of exposure (mean ± SD; $n = 5$). Different letters (a–c) indicate significant differences between treatments (for the same tissue; $p < 0.05$). Abbreviations: Control – reference temperature and pH conditions (i.e. T = 19 °C and pH = 8.0 units); TCS – triclosan exposure; Acid – simulated acidification (i.e. pH = 7.6 units); Warm – simulated warming (i.e. T = 24 °C), CAT – catalase activity; SOD – superoxide dismutase inhibition; GST – glutathione S-transferase; LPO – lipid peroxidation.

protein synthesis in fish muscle (i.e. 85.4% and 66.0% increase in relation to Control treatment in Acid + Warm and TCS + Warm treatments, respectively; $p < 0.001$ in both cases; Fig. 5D and *Supplementary Materials 1*_Table S7). On the other hand, Ub contents in fish liver were significantly increased in acidified conditions (i.e. average increase of over 100% in relation to the Control treatment for TCS + Acid and TCS + Acid + Warm treatments; Fig. 5E and *Supplementary Materials 1*_Table S7). In the brain, with the exception of Acid + Warm and TCS treatments, all other treatments significantly reduced Ub protein synthesis in relation to the Control treatment, particularly with acidification acting alone (which yielded a 69.5% decrease in relation to Control treatment; $p < 0.001$; Fig. 5F and *Supplementary Materials 1*_Table S7). Ub contents in both liver and brain of contaminated fish were significantly correlated with TCS contents ($r = 0.47$ and $r = 0.51$, respectively, and $p < 0.050$ in both cases), as well as to K (only in liver; $r = -0.33$ and $p < 0.050$; Table 2).

AChE brain activity and VTG liver content are shown in Fig. 6. A significant inhibition of AChE brain activity was observed in Warm, Warm + Acid, TCS and TCS + Acid + Warm treatments (activity decreases between 22.4% and 28.1% in relation to Control treatment; $p < 0.001$ in all cases), while no significant effects were observed in Acid, TCS + Warm and TCS + Acid treatments ($p > 0.050$; Fig. 6A and *Supplementary Materials 1*_Table S7). Furthermore, this AChE activity inhibition was not correlated with TCS brain concentrations, nor with animal fitness indexes (K and BB_{ratio}). As for VTG liver content, the combination of acidification and warming, as well as TCS exposure (regardless of temperature and pCO_2) significantly reduced the production of this protein, with TCS + Acid and TCS + Acid + Warm treatments registering the highest inhibition in relation to the Control treatment (i.e. a decrease around 80% in relation to Control treatment; $p < 0.001$ in both cases; Fig. 6B and *Supplementary Materials 1*_Table S7). In addition, a significant negative correlation found between VTG content and K ($r = 0.37$ and $p < 0.050$; Table 2).

4. Discussion

The present study provided relevant and innovative findings showing that TCS bioaccumulation mechanisms in marine fish

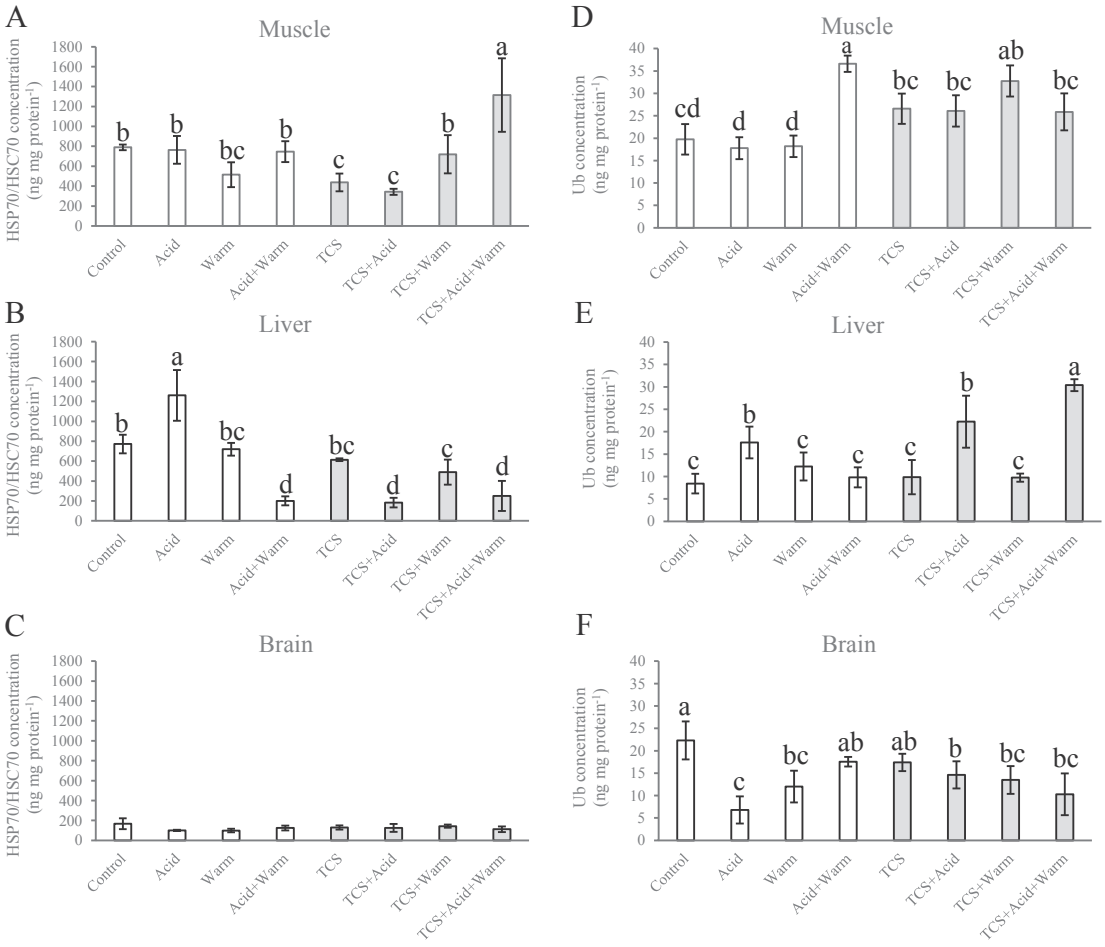

Fig. 5. HSP/HSC70 (μg mg^{-1} protein) and ubiquitin (Ub; μg mg^{-1} protein) concentrations in muscle (A, D), liver (B, E) and brain (C, F) of *D. sargus* after 28 days of exposure (mean ± SD; $n = 5$). Different letters (a–d) indicate significant differences between treatments (for the same tissue; $p < 0.05$). Abbreviations: Control – reference temperature and pH conditions (i.e. T = 19 °C and pH = 8.0 units); TCS – triclosan exposure; Acid – simulated acidification (i.e. pH = 7.6 units); Warm – simulated warming (i.e. T = 24 °C); HSP70/HSC70 – heat shock proteins; Ub – total ubiquitin.

species are influenced by the surrounding abiotic conditions and that increased temperature and pCO_2 levels can be generally associated with lower TCS tissue burdens. As for the ecotoxicological responses of juvenile *D. sargus*, TCS dietary exposure significantly increased LPO, as well as decreased AChE brain activity and VTG liver content, but less evident effects were observed in fish antioxidant machinery (CAT, SOD and GST activities). Furthermore, these effects were either exacerbated or attenuated/reversed by the co-exposure to abiotic stressors, with the combination of TCS and acidification resulting, overall, in more notorious biochemical alterations (namely, in SOD, LPO, HSP70/HSC70, Ub and VTG) compared to the other treatments.

4.1. TCS concentration in feeds, seawater and non-contaminated fish samples

TCS was efficiently incorporated in TCS-contaminated feed, evidencing a stable concentration during the whole trial (i.e. TCS in feed did not undergo degradation/loss throughout time). The fact that TCS was not detected in CTR feed indicates that no external contamination occurred during feed preparation. Furthermore, seawater and fish samples from Control treatment also revealed TCS levels below LOD, thus, confirming that during the trial no external contamination has taken place within the independent recirculation aquaculture system, apart from the intended TCS dietary exposure in contaminated treatments. In this way, the quantifiable (and similar among treatments) TCS levels in seawater samples from contaminated treatments after 28 days of dietary exposure to TCS can only be attributed to two reasons: i) TCS partial leaching from contaminated feed to seawater, which seems less likely to have occurred given the physical and chemical properties of this compound (i.e. low water solubility and high adsorption to solid particles; Dhillon et al., 2015); and ii) faecal excretion by fish of TCS in its parental form (Escarrone et al., 2016; Arnot et al., 2017). It should be noted that the low, but not negligible, TCS levels in seawater from contaminated treatments, to which fish were chronically exposed during the trial, might have also contributed (even if to a much lower extent compared to the intended dietary exposure) to the final TCS tissue burden due to its re-uptake through fish gills (i.e. via inhalation), possibly enhancing the ecotoxicological effects elicited by the dietary exposure to this compound (as well as the studied abiotic stressors).

4.2. TCS tissue bioaccumulation and effects of warming and acidification

Even though PPCPs are generally assumed to be mostly uptaken by marine biota through direct contact with contaminated water

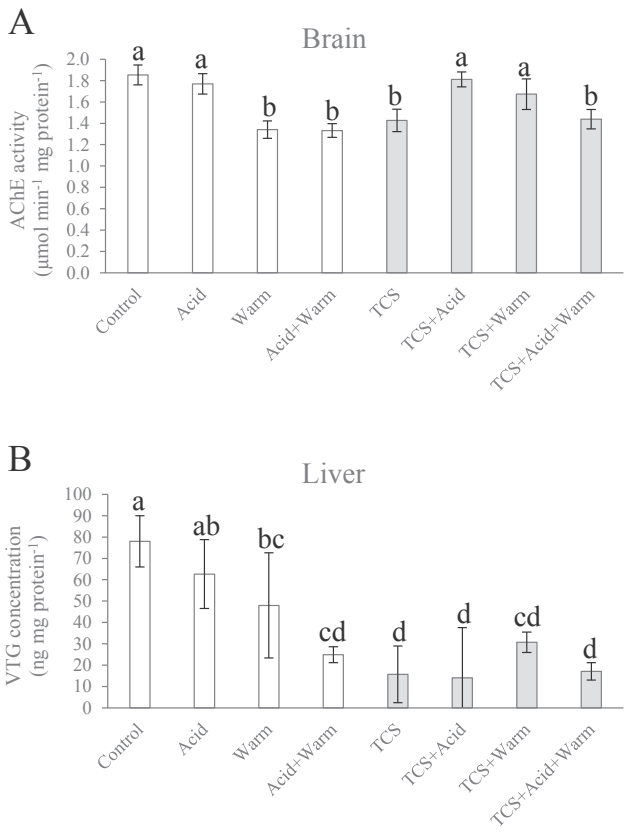

Fig. 6. Acetylcholinesterase (AChE) activity (µmol min $^{-1}$ mg^{-1} protein; 4A) in brain tissues and vitellogenin (VTG) concentration (ng mg^{-1} protein; 4B) in liver tissues of *D. sargus* after 28 days of trial (mean ± SD; $n = 5$). Different letters (a–d) indicate significant differences between treatments ($p < 0.05$). Abbreviations: TCS – triclosan exposure; Acid – simulated acidification (i.e. pH = 7.6 units); Warm – simulated warming (i.e. T = 24 °C); AChE – acetylcholinesterase activity; VTG – vitellogenin concentration.

masses (i.e. via gills through inhalation, or via skin), recent studies have pointed out dietary transfer (i.e. biomagnification along the food webs) as an important pathway for contaminants' bioaccumulation in marine fish species, particularly for organic and/or less hydrosoluble compounds (e.g. Dijkstra et al., 2013; Zenker et al., 2014; Maulvault et al., 2016, 2018b). Hence, even though the TCS exposure concentration selected in study may not mimic a dietary exposure that occurs in nature (e.g. TCS levels in bivalve species, which are natural preys of *D. sargus*, are around 2 ng g^{-1} dw, i.e., ~7 times lower than TCS concentration in the contaminated feed; Álvarez-Muñoz et al., 2015), the present results constitute a proof of concept that, indeed, TCS can be uptaken by marine fish species through diet, with muscle being the primary organ for bioaccumulation of this compound. To date, studies on TCS bioaccumulation in different fish tissues are extremely limited (Escarrone et al., 2016) and, to the best of the authors' knowledge, no such studies have been carried out using marine species exposed to TCS via diet, thus, hindering comparisons of the present results with previous reports. Yet, the lipophilic behaviour of TCS suggests that it can be highly bioaccumulative and persistent in fatty tissues, such as liver and gonads, while much lower bioaccumulation is expected to occur in tissues with lower lipid content, such as muscle and brain (Dhillon et al., 2015; Escarrone et al., 2016). Indeed, Escarrone et al. (2016) detected higher TCS concentrations in fish gonads and liver, and lower levels in gills, brain and muscle after 14 days of TCS water exposure (160 µg L^{-1}). Such findings contradict the pattern observed in the present study (i.e. muscle TCS concentrations were ~10 times higher than those of liver, under the reference temperature and pCO_2 conditions), suggesting that contaminant exposure route, dose and time may play a preponderant role on TCS's mechanisms of bioaccumulation and tissue distribution in fish. On the other hand, the surprisingly lower persistence of TCS in its parental form observed in the liver could be due to an intensive compound biotransformation that might have occurred in this tissue, since liver is the primary organ for the metabolization and detoxification of xenobiotics, being then transported to other organs through the blood circulation or excreted via the hepatobiliary route (Wang et al., 2010; Maulvault et al., 2016). The toxicokinetic mechanisms of TCS in fish species are still poorly understood, however, recent data have shown that TCS is intensively and rapidly metabolized in the liver, where it partially undergoes glucuronidation or sulfonation, being subsequently transformed into more soluble and easily excreted metabolites (James et al., 2012). Such TCS detoxification mechanism seem to have been enhanced by seawater warming and acidification (although, to a lower extent in this last case), substantially reducing TCS levels in all tissues.

Warmer temperatures often enhance fish metabolism, which translate into increased ventilation and feeding rates in order to fulfil the higher metabolic demands (Rosa et al., 2013, 2014; Anacleto et al., 2018). Thus, although an enhanced animal metabolism can result in higher contaminants' uptake (of those dissolved in the water column, i.e. via respiration, or those present in the feed, i.e. via ingestion; e.g. Dijkstra et al., 2013; Hedrick-Hopper et al., 2015; Maulvault et al., 2016, 2018b), it can also facilitate contaminants' metabolization and elimination (Maulvault et al., 2016, 2018b; Serra-Compte et al., 2018). Moreover, increased metabolic rates can enhance animal growth, which can also translate into lower contaminant bioaccumulation due to somatic growth dilution (Dijkstra et al., 2013; Maulvault et al., 2016). This argument may also explain the lower TCS tissue burden in fish exposed to warming conditions, which match the generally higher W and TL also found in these treatments, as well as the negative correlation observed between these parameters and TCS concentrations in liver and brain of contaminated fish. As follows, here we argue that the enhanced metabolism of *D. sargus* elicited by 28 days of exposure to +5 °C of seawater temperature could have been accompanied by an increase in both animal growth and TCS metabolization/excretion, therefore, translating into lower TCS tissue burdens in fish under warming. Yet, such pattern seemed to have been somewhat reversed by acidification in fish muscle, as TCS + Acid + Warm treatment showed higher TCS concentrations than TCS + Warm treatment, but similar values to those observed in TCS + Acid treatment.

Contaminants' chemical properties are largely influenced by environmental conditions, with metals and other ionic compounds being particularly affected by the surrounding seawater pH levels. Such is the case of TCS that becomes increasingly protonated and loses its negative charge as pH falls below ~8.0 units (Orvos et al., 2002; Rowett et al., 2016). In this way, a recent study using the freshwater amphipod *Gammarus pulex* as model organism evidenced increased TCS toxicity under lower pH levels (i.e. 7.3 pH units *versus* 8.4 pH units), and such results were justified by the fact that cell lipid membranes are generally impermeable to ionized molecular forms (i.e. ionized TCS forms are less toxic; Rowett et al., 2016). Yet, the present results are not in agreement with this argument, since fish exposed to acidification alone also exhibited lower TCS tissue burdens than those under normal temperature and pCO_2 conditions, though not as much as warming. Such results can be explained by the three following points. First, the study of Rowett et al. (2016) was focused on TCS exposure via water and,

therefore, a greater influence of the surrounding pH level on TCS's availability can be expected in this case, in comparison with the conditions simulated in the present study (i.e. TCS exposure via feed). Second, in this previous study, a higher ΔpH was simulated (i.e. a decrease of −0.9 pH units, whereas in the present study ΔpH = −0.4 pH units), as more pronounced changes in pH levels can be expected in freshwater environments likely conditioning the ratio between molecular and ionized TCS forms available for fish uptake to a greater extent than the one elicited in the present study. Third, the energy required to maintain animal homeostasis under −0.4 pH units could have diminished fish biological activities, thus, reducing the uptake rates of TCS. It is also worth mentioning that the trends observed in fish liver and brain under acidification were further enhanced when warming was also added to the equation (i.e. lower values in TCS + Acid + Warm treatment compared to TCS + Acid treatment), and that was certainly related to the increased metabolic rates (and, the corresponding enhanced TCS detoxification) induced by +5 °C of seawater temperature.

Linking the present findings with environmental and seafood safety, despite results do not evidence increased TCS uptake in fish tissues under warming and/or acidification, the trends presently observed clearly confirm that PPCPs' fish tissue burdens are strongly influenced by seawater abiotic variables, which can alter compounds' bioaccumulation, metabolization and elimination mechanisms. Hence, such findings highlight that the interactive effects of abiotic variables should not be neglected when estimating the environmental hazards posed by PPCPs, nor when regulating the presence of PPCPs in the environment and seafood species. Furthermore, the current lack of literature on this matter, along with the controversial results reported in the few available studies (Hedrick-Hopper et al., 2015; Escarrone et al., 2016) calls for the need to further investigate the impacts of climate change from an ecotoxicological point of view, considering different contaminant exposure routes, as well as exploring less pronounced to more severe ranges of stressors' effects.

4.3. Interactive ecotoxicological effects of TCS, warming and acidification in D. sargus

Although animal condition can gradually decline when fish are exposed to environmental stressors due to additional energetic costs that prevent the allocation of energy resources towards somatic growth (Sandblom et al., 2016; Maulvault et al., 2018a; Anacleto et al., 2018), juvenile *D. sargus* did not exhibit significant differences in K (nor in BB_{ratio}) after 28 days of exposure to the experimental conditions. Therefore, results suggest that fish physiological mechanisms were somewhat able to compensate the stress induced by TCS, +5 °C and/or +1000 μatm pCO_2 (equivalent to −0.4 pH units) during 28 days of exposure trial. On the other hand, fish exposed to TCS alone or combined with acidification revealed, in overall, higher HSI than the other treatments (though statistical significance was only obtained against TCS + Acid + Warm treatment). These results match the trends observed in TCS tissue concentrations (i.e. higher TCS concentrations in TCS and TCS + Acid treatments, along with the positive correlation found between TCS liver concentration and HIS), and are in agreement with the fact that xenobiotics' exposure usually provokes liver hypertrophy and/or hyperplasia (Diniz et al., 2009; Sadekarpawar and Parikh, 2013; Maulvault et al., 2017). Conversely, the significantly lower HSI observed in fish exposed to the combination of the three stressors (compared to TCS exposure alone or combined with acidification) may be associated with the lower TCS liver concentration elicited in this treatment and/or with cell apoptosis promoted by such severe stress conditions.

In agreement with previous findings (e.g. Canesi et al., 2007; Matozzo et al., 2002; Liang et al., 2013; Maulvault et al., 2017, 2018a), the exposure to the three studied stressors resulted in both up- and down regulations of molecular biomarker responses, according to fish tissue and biomarker. The differential tissue responses (e.g. higher CAT activity in muscle than in liver and brain; and CAT muscle activity induced by TCS alone, whereas CAT liver activity was inhibited by TCS plus acidification) were likely related to the fact that different tissues not only can have distinct baseline levels of molecular biomarkers, but may also respond differently to the presence of stressors (such as, TCS, warming and/or acidification), since each tissue is composed by different cell types (i.e. organs are physiologically and functionally different). Hence, the current findings highlight the importance of assessing fish tissue differential responses in ecotoxicological studies, as such approach provides a broader view on the impacts of environmental stressors in a whole organism context.

The antioxidant enzymes SOD, CAT and GST play a key role in cells' defence mechanisms against the oxidative stress induced by environmental stressors, i.e.: SOD converts superoxide radicals into hydrogen peroxide and molecular oxygen (O_2) and, then, CAT converts hydrogen peroxide into oxygen and water (Halliwell and Gutteridge, 1985), while GST is a major second phase detoxification enzyme (Sheehan et al., 2001), being also responsible for generating less toxic and more hydrophilic compounds through the conjugation of lipid peroxidase breakdown products with glutathione (GSH; Barata et al., 2005; Park et al., 2017). In this way, previous studies conducted in marine organisms have reported an induction of CAT, SOD and GST activities under increased temperature and pCO_2 levels (e.g. Rosa et al., 2016; Jesus et al., 2018; Maulvault et al., 2017, 2018a), as well as under chemical contaminants' exposure (e.g. Matozzo et al., 2002; Maulvault et al., 2017, 2018a), thus, corroborating the findings obtained with *D. sargus* exposed to warming and/or acidification (increased GST brain activity), TCS (increased CAT muscle activity, as well as a positive correlation between TCS concentration and CAT activity in contaminated fish muscle) and TCS co-exposed with acidification (increased SOD liver and brain activities). In agreement, Park et al. (2017) reported increased SOD and GST activities in the copepod *Tigriopus japonicus* short-term exposed to different concentrations of TCS via water (50 and 100 $\mu g\,L^{-1}$). Similarly, in response to TCS water exposure, Matozzo et al. (2002) found a significant increase in SOD gills activity in *Ruditapes philippinarum*, while no changes were observed in CAT activity. Yet, an opposite effect was also observed in our study, with CAT (liver) and GST (muscle and liver) activities being inhibited by the co-exposure of TCS and warming, though such inhibition seemed to have been somewhat attenuated by acidification. Such pattern suggests that, despite the lower TCS levels in TCS + Warm treatment in the different tissues, the severity of stress induced by the combination of TCS exposure and +5 °C must have led cells' antioxidant mechanisms to exhaustion, possibly due to an excessive production of H_2O_2 (Gonzalez-Rey and Bebianno, 2014; Maulvault et al., 2018a). SOD muscle activity was also inhibited in Warm and Acid + Warm treatments, most likely due to ROS-mediated denaturation promoted by increased temperatures resulting in the inactivation of this enzyme (Ferreira et al., 2015), but such effect was reversed by TCS co-exposure. Though literature is extremely scarce in what concerns the interactive effects of PPCPs and abiotic variables on marine species ecotoxicological responses (with no studies being focused on cells' antioxidant activity, to the best of the authors' knowledge), a similar trend was also observed in our recent study performed with *Dicenthrachus labrax*, where the increased SOD inhibition in fish muscle promoted by warming and acidification was partially attenuated by diclofenac co-exposure (Maulvault et al., 2018a).

In line with the enhanced CAT and SOD activities, TCS exposure alone or combined with acidification elicited the formation of lipid peroxides in fish muscle and liver (i.e. increased MDA concentration in relation to the remaining treatments), a result that is most likely related with the higher TCS levels found in fish exposed to these conditions. The increased LPO found in these treatments suggests that the enhancement of antioxidant defences was only able to prevent the oxidative stress induced by TCS and acidification to some extent, leading to the consequent cell damage.

Matching some of the present findings (i.e. HSP70/HSC70 content in fish liver under acidification and muscle of fish exposed to the three stressors alone), the synthesis of molecular chaperones, such as HSPs, can be induced in order to repair, refold, and/or eliminate damaged proteins, thus preventing cellular damage promoted by the exposure to both abiotic stressors (e.g. Rosa et al., 2014; Pimentel et al., 2015; Madeira et al., 2016a,b) and chemical contaminants (e.g. Maulvault et al., 2017, 2018a), including TCS (Lin et al., 2014). The positive correlation found between TCS and HSP70/HSC70 concentrations in this tissue is consistent with this trend, thus evidencing that chaperoning mechanisms were further enhanced as TCS tissue levels increased (and *vice-versa*). Yet, chaperone activation mechanisms cannot be looked at as straightforward process, as they are influenced by several physiological factors, including stress levels, interactions with chemical contaminants, species, tissue and animal hormone levels, (e.g. Mahmood et al., 2014; Madeira et al., 2016b). Furthermore, HSPs can also be down-regulated due to the exhaustion of cytoprotective systems and metabolic depression promoted by severe and/or chronic stress conditions (Madeira et al., 2016b; Maulvault et al., 2018a). Such was the case of fish liver exposed to acidification and/or warming (regardless of TCS exposure), as well as muscle of fish exposed to TCS and acidification. Similarly, a previous study with *D. labrax* reported that HSP70/HSC70 synthesis in fish muscle was up-regulated by acidification and warming in co-exposure with diclofenac, but down-regulated by acidification alone (Maulvault et al., 2018a). Falisse et al. (2017) also reported HSPs overexpression in zebrafish (*Danio rerio*) larvae following TCS seawater exposure at 100 $\mu g\,L^{-1}$, but not at 50 $\mu g\,L^{-1}$, suggesting that TCS mode of action may be dose-dependent.

Similar to HSPs, Ub synthesis can be induced when internal stress is generated, in order to signal denatured proteins to be degraded by the proteasome (e.g. Gravel, and Vijayan, 2007; Madeira et al., 2017). This pattern was observed in fish liver exposed to increased pCO_2 levels (i.e. Acid, Acid + Warm, TCS + Acid and TCS + Acid + Warm treatments), where increased Ub levels were generally negatively correlated with lower K. Furthermore, results also indicate that the synthesis of these proteins was positively correlated with TCS levels in liver and brain. Conversely, extreme or long-lasting stress conditions can also result in Ub inhibition due to physiological collapse, since protein synthesis is an energy demanding process, requiring over 50% of all O_2 consumed my fish (e.g. Gravel, and Vijayan, 2007; Madeira et al., 2016b). Indeed, the overall decrease Ub content observed in fish brain suggests that the exposure to TCS, acidification and/or warming led to irreversible damages, that both chaperoning and ubiquitin-proteosomal pathways could not compensate for, most likely as a result of a poorer energy budget (and aerobic scope) in fish under these stressors (Falfushynska et al., 2014; Madeira et al., 2017). Moreover, Ub inhibition was particularly evident under acidification alone or combined with TCS and warming, and that may be related to the fact that elevated pCO_2 levels disrupt the ionic balance in proton-based neurotransmitter cell receptors (e.g. $GABA_A$), with the consequent increase of animal stress and cognition impairments (e.g. Nilsson et al., 2012; Hamilton et al., 2014; Munday et al., 2014; Maulvault et al., 2018c).

AChE was also inhibited by warming alone or by the co-exposed with acidification, and by TCS alone or by the co-exposure to the two abiotic stressors simultaneously. These results are in line with previous findings: i) the exposure to abiotic stressors, particularly increased pCO_2 levels, strongly mediates AChE brain activity (Rosa et al., 2016); and ii) AChE can be a target for chemical contaminants, leading to the inhibition of its activity and, therefore, to failed synaptic transmission and muscle overstimulation (Schmidel et al., 2014; Maulvault et al., 2017; Topal et al., 2017). Furthermore, AChE activity inhibition seemed to be consistent with the Ub synthesis impairment in the brain, particularly in TCS-exposed treatments, suggesting that TCS dietary exposure may have a neurotoxic effect in fish species. In accordance with the present results, Hedrick-Hopper et al. (2015) observed impaired reflexes in Atlantic croaker (*Micropogonias undulatus*) following TCS dietary exposure, with the dorsal fin response being further affected by the combination of TCS and increased temperatures, evidencing that TCS's toxicological attributes may be accentuated by the predicted climate change seawater conditions. It is also worth noting that Falisse et al. (2017) reported acute neurotoxicological effects in *D. rerio* larvae exposed to TCS (50 and 100 $\mu g\,L^{-1}$), though such effects translated into an induction of AChE activity rather than an inhibition, contrasting with the present findings.

VTG is usually very low or undetectable in male and juvenile individuals, but can reach high values in the liver and plasma of mature females. Nonetheless, the exposure to endocrine disrupting compounds has been previously described to either elicit its production in males/juveniles (estrogenic effect) or inhibit its production in females (antiestrogenic effect; Matozzo et al., 2008; Gonzalez-Rey and Bebianno, 2014), causing remarkable reproductive impairments and, ultimately, deleterious ecological consequences. Such impacts may be even more severe in protandrous fish, such as those belonging to the *Sparidae* family, which can alternate from one sex to another throughout its life cycle. This is the case of *D. sargus*, which is a digynic hermaphrodite species, i.e. some early life-stage individuals exhibit non-functional bisexual gonads, which can develop into male or female due to various environmental factors (e.g. demographics, social behaviour, abiotic conditions and insufficient nutrition; once mature, some males retain the ability to invert sex into secondary females (Morato et al., 2003). Moreover, since marine fish species' reproductive strategies are strongly mediated by the surrounding abiotic conditions, warming and acidification are also expected to affect their reproduction and ecological success, or even to accentuate their negative responses to the presence of endocrine disrupting compounds (e.g. Morato et al., 2003; Pankhurst and Munday, 2011; Cripps et al., 2014). These arguments are in accordance with the present findings, since TCS dietary exposure drastically inhibited VTG synthesis in relation to the values observed under control conditions and, therefore, showed a potential antiestrogenic effect in juvenile *D. sargus*, as did warming and acidification (except when these last two acted alone). Interestingly, the greatest inhibition was observed under the combination of TCS and increased pCO_2 levels (i.e. TCS + Acid and TCS + Acid + Warm treatments). These results follow the trend observed in an earlier study with *D. labrax*, i.e. acidification and/or warming also seemed to have an antiestrogenic effect (i.e. lowering VTG levels) by counteracting the effects of dietary exposure to diclofenac (Maulvault et al., 2018a). Even though the effects of increased temperatures are, by far, better described in literature (e.g. Chandra et al., 2012; Hedrick-Hopper et al., 2015; Maulvault et al., 2018a), previous studies also pointed out that acidification can play a preponderant role on marine biota's endocrine regulation (e.g. by modulating the synthesis of cortisol levels and thyroid hormones, which are, in turn, linked to VTG induction; McCormick and Bradshaw, 2006; Kwong et al., 2014). Yet,

little is still known regarding interactive effects of co-exposure to acidification and endocrine disruptors, thus calling for the need to further investigate the effects of environmental stressors on fish endocrine system and reproduction.

5. Conclusions

Data confirmed that TCS uptake and elimination mechanisms in the marine fish species *D. sargus* are strongly influenced by seawater temperature and pCO_2 levels, and that the metabolic and physiological changes elicited by warmer and/or more acid environmental conditions can result in lower TCS tissue burdens.

As for ecotoxicological responses, results showed that TCS dietary exposure promoted cellular damage (increased LPO), neurotoxicity (AChE inhibition) and endocrine disruption (VTG inhibition) in juvenile *D. sargus,* while its effect on tissues' antioxidant machinery (CAT, SOD and GST activities) was not so clear. Yet, the differential responses (up- or down-regulation) observed according to tissue pointed out that TCS can have distinct modes of action depending on animal tissue and enzyme. TCS toxicological attributes were also largely influenced by increased temperature (+5 °C) and pCO_2 levels (+1000 μatm), resulting in an enhancement or attenuation of biochemical biomarker responses. Overall, TCS co-exposed with acidification resulted in more drastic biochemical alterations than those elicited by TCS co-exposed with warming or even the combination of the three stressors, resulting in increased SOD activity and LPO, as well as in diminished chaperoning and VTG synthesis. Hence, the distinct effects promoted by TCS exposure, acidification and warming, acting alone or combined, highlight the need to consider the interactions between environmental stressors in future ecotoxicological studies, as well as to incorporate these variables in future regulations and recommendations regarding the environmental presence of PPCPs. Such actions will be crucial to understand and mitigate the toxicological implications of climate change, as environmental stressors unlikely occur in isolation, nor the alterations of environmental conditions affect marine ecosystems in the same way across the planet.

Ethical statement

Fish trials were performed according to European regulations (EU Directive 2010/63), and previously approved by the Ethical Committee of the Faculty of Sciences of the Lisbon University. All researchers involved in fish maintenance, handling and sampling were previously accredited by the Federation of European Laboratory Animal Science Associations (FELASA).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2018.11.020.

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